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ANTI-INFLAMMATORY ACTIVITY, HPTLC ANALYSIS OF β-SITOSTEROL FROM METHANOLIC LEAF EXTRACTS OF *THUNBERGIA GRANDIFLORA* ROXB., TRADITIONAL HERBAL PLANT FROM MIZORAM, INDIA

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Keywords:

Thunbergia grandiflora Roxb., Anti-inflammatory, HPTLC,β -sitosterol

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ABSTRACT: *Thunbergia grandiflora* Roxb. (Mizo local name- Vakohrui or Zawngafian), commonly known as Bengal clockvine, is a long-lived, dicotyledonous plant traditionally used in the state of Mizoram, India for the treatment of cancer, diabetes, eye diseases, cuts and wounds *etc*. This study aimed to conduct *in-vitro, in-vivo* anti-inflammatory studies and HPTLC fingerprinting and quantifying the bioactive compounds from *Thunbergia grandiflora* Roxb. The IC_{50} value of Egg albumin and BSA denaturation studies was found to be 423.59 µg/ml & 400.76 µg/ml respectively. The results showed that methanolic leaf extract of the plant 200mg/kg b.w. and 400mg/kg b.w. considerably reduced carrageenan-induced paw edema, and their effects were comparable to the standard diclofenac sodium. HPTLC analysis showed the presence of β -Sitosterol in the methanolic extract and the amount of β -sitosterol was found to be 22ng/µl. The identity of the bands of β -Sitosterol in the samples was confirmed by overlapping their UV absorption spectrum with that of the standards. The results for anti-inflammatory studies showed marked anti-inflammatory activity, which was found to be concentration dependent. These results suggested the methanolic leaf extract of *Thunbergia grandiflora* plant of having promisinganti-inflammatory properties that could, in the future aid the pharmaceutical industry in the formulation of safe, herbal anti-inflammatory drugs.

INTRODUCTION:

***Thunbergia grandiflora* Roxb.:** Inflammation is frequently associated with pain, making the site complex. Increased vascular permeability, protein denaturation, and membrane alteration are occurrences of inflammation [1].

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Prescription of over-the-counter (OTC) drugs such as nonsteroidal anti-inflammatory drugs (NSAID) or corticosteroids pain relievers are recommended to reduce pain. Unfortunately, short-term or long-term negative side effects such as bleeding, indigestion, heart problems, and kidney issues persist in some of these drugs.

Due to this, more patients are turning to natural products to manage pain, which results in increased efforts to develop natural anti-inflammatory medicines [2]. This search is worthwhile due to the perceived efficacy, low incidence of major side

effects or relative safety compared to the synthetical alternatives, and the accessibility of medications derived from plants [3]. High-Performance Thin Layer Chromatography (HPTLC) is an improved and sophisticated form of Thin Layer Chromatography (TLC) [4]. It is based on the principle of adsorption. The mobile phase solvent flows through because of capillary action. The components separate according to their affinities. The component with more affinity towards the stationary phase travels slower, while the component with more affinity towards the mobile phase travels faster [5]. Compared to the classical TLC, HPTLC is a simple, fast and accurate technique for use, which makes it advantageous for quick assessment of several samples simultaneously [6]. HPTLC is more advantageous in ways like improved sample application (automatic or semi-automatic), higher separation efficiencies, less mobile phase usage, automatization of the drying of the plates, less time required for analysis *etc*.[7].

Thunbergia grandiflora Roxb. (Mizo local name-Vakohrui or Zawngafian) is commonly a long-lived, dicotyledonous plant widely distributed in tropical and subtropical regions. Traditionally, in Mizoram, India, it is used for treating cancer, diabetes, eye diseases, cuts, and wounds.

MATERIALS AND METHOD:

Collection and Authentication of Plant: *Thunbergia grandiflora* Roxb leaves were collected from Mamit District, the southern part of Mizoram during August 2021. A herbarium sheet of the plant material was prepared and sent to the Botanical Survey of India, Eastern Regional Centre, Shillong for authentication. The given identification number is BSI/ERC/Tech/2021/199.

Experimental Animals: Wistar Albino rats (150–200g) of either sex were used in this study. The animals were kept in the animal house of the Department of Pharmacy, Regional Institute of Paramedical and Nursing Sciences. The animals were housed in large propylene cages at 22±2°C in 12 h dark-light cycle and were fed with rat pellet food and water *ad libitum*. All animals were acclimatized for at least one week before the experimental session. All the experimental procedures followed the guidelines of the Institutional Animal Ethics Committee (IAEC) after approval (Approval no: IAEC/RIPANS/79).

Preparation of Extracts: *Thunbergia grandiflora* Roxb. plant materials were air-dried in the shade in a dark room. It was then grinded into coarse powder, sieved and subjected to successive soxhlet extraction using solvents based on the increasing polarity order conducted. The plant materials were extracted with petroleum ether, chloroform, and methanol. The extracts were then evaporated using rotary vacuum evaporator and stored in the refrigerator for further analysis.

Preliminary Phytochemical Screening: Preliminary phytochemical screening data of *Thunbergia grandiflora* Roxb. leaf extract was already published in literature [8].

Acute Toxicity Test: The results of the acute toxicity tests for *Thunbergia grandiflora* Roxb. extracts were taken from previously published literature [9], where all selected doses were deemed to be healthy with no mortality by evaluating dosage of up to 2000 mg/kg protection in accordance with OECD 423 guidelines.

Anti-Inflammatory Activity (*In-vitro*):

Egg Albumin Denaturation Method: Egg albumin denaturation study was done as per the earlier reported method [10]. The reaction mixture (5 mL) consisted of 0.2 ml of egg albumin (from fresh hen's egg), 2.8 ml phosphate buffered saline (pH 6.4) and 2mL of varying concentrations (100,200,300,400,500 μg/mL) of plant extracts. Similarly, control was made for reference by using distilled water. Diclofenac sodium was used as the reference drug and treated similarly to determine absorbance. The mixtures were then incubated at 37±2ºC in an incubator for 15 minutes and then heated at 70ºC for 5 minutes. After cooling, the absorbance was measured at 660 nm by using UV-visible spectrophotometer. The vehicle was used as blank. The Percentage inhibition of protein denaturation was calculated by using the formula:

$$Percentage\ inhibition\ (\%) = 100 - (\text{Abs of control - Abs of sample}) / \text{Abs of control} \times 100 \quad .... \text{(Eq 1)}$$

Bovine Serum Albumin Denaturation Method: Bovine Serum Albumin (BSA) denaturation assay of the methanolic extract of *Thunbergia*

grandiflora Roxb. was determined using a modified version of the earlier reported method [10], 5% w/v aqueous solution of BSA was prepared. The test solution (0.5 mL) consisted of 0.45 mL of BSA and 0.5 ml of the test solution (100, 200, 300, 400, 500 µg/ml). Similarly, control was made for reference by using distilled water. The standard solution (0.5 ml) consisted of 0.45 ml of BSA (5% w/v aqueous solution) and 0.5 ml of diclofenac sodium (100, 200, 300, 400, 500µg/ml).

The samples were then incubated at 37°C for 20 minutes, and the temperature was increased to keep the samples at 57°C for 3 minutes. After cooling, 2.5 ml of phosphate-buffered saline was added to the above solutions. The absorbance was measured using a UV-visible spectrophotometer at 416 nm. The vehicle was used as blank. The Percentage inhibition of protein denaturation was calculated by using (Eq 1).

Anti-inflammatory Study (*In-vivo*):

Carrageenan-Induced Paw Edema: Previously reported procedures were followed with slight modifications to study the carrageenan-induced paw edema of the methanol extracts of *Thunbergia grandiflora* Roxb [11, 12].

The rats were randomly divided into four groups of four animals. Group I was kept as the control group, receiving just distilled water. Test substance METG was administered orally to Groups III and IV at doses of 200 and 400 mg/kg body weight, respectively. In contrast, Group II was given a treatment of diclofenac (10mg/kg) as the reference standard for comparison. 1% carrageenan was injected into each animal's right hind paw, an hour after the oral administration of the test materials. Following the carrageenan administration, the volume of paw edema was measured at 0, 1, 2, 3, 4 and 5 hours using digital Vernier caliper. The left hind paw served as a reference non- inflamed paw for comparison. The average percent increase in paw volume with time was calculated and compared against the control group.

The percent inhibition was calculated using the formula:

$$\text{Percentage inhibition (\%)} = (Vc\text{-}Vt) / Vc \times 100$$

Vc and Vt represent the average paw volume of control and treated animals, respectively.

HPTLC Finger Printing: A sample working solution of 30 mg/mL concentration of the methanolic extract of *Thunbergia grandiflora* Roxb. Was prepared by dissolving 150mg of the plant extract in 5mL of methanol, a stock solution of the standard β -Sitosterol of 1000 µg/mL concentration was prepared. A standard working solution of 40 µg/mL concentration was prepared by taking 40µL of the stock solution and adding 960µL of methanol.

The mobile phase was prepared by mixing petroleum ether, ethyl acetate, and acetonitrile in the volume ratio of 8.2: 1.8: 0.1, previously reported [13]. During development of each plate, a fresh mobile phase was prepared. Bands of the sample (4µl, 6 µl and 8µl) and standard (2µl and 4µl) were applied in duplicate on pre-coated TLC silica gel 60 F254 aluminum sheets (10x10 cm) with the help of Linomat 5 applicator connected to CAMAG-HPTLC system programmed through Wincats software. After the sample application, the chromatogram was developed in a Twin trough glass chamber (10x10 cm) saturated with the developed solvent for 20 minutes.

Dilute Sulphuric acid reagent was used as a post-derivatizing reagent by taking 10 mL of sulphuric acid, which was gradually added to 70mL ice-cooled distilled water in a 100 mL volumetric flask. The volume was made up of distilled water. The air-dried plate was viewed in ultraviolet radiation to mid-day light. The developed plate was documented using a digital documentation system under UV light at 366 nm and 254 nm. CAMAG TLC Scanner scanned the chromatogram for densitometric evaluation of HPTLC Chromatograms. The fingerprint data was recorded by WINCATS software

HPTLC Quantification: HPTLC quantification of METG along with standard β-Sitosterol was performed by developing the plate in a Twin trough chamber saturated with the solvent system petroleum ether: ethyl acetate: acetonitrile (8.2: 1.8: 0.1) for 20 minutes. The plates were then scanned at 366 nm and 254 nm. CAMAG TLC Scanner scanned the chromatogram for densitometric evaluation of HPTLC Chromatograms. The fingerprint data was recorded by WINCATS software.

RESULT:

Anti-inflammatory Activity (*In-vitro*):

Egg Albumin Denaturation Method: The percentage inhibition of egg albumin denaturation of the extract was compared with the standard diclofenac sodium. The study was performed in triplicate; the percentage inhibition and the drug response comparison are given in **Table 1**. The IC_{50} of *Thunbergia grandiflora* Roxb. was found to be 423.59µg/ml as compared to the standard diclofenac sodium, which was found to be 313.58µg/ml. The maximum inhibition of *T. grandiflora Roxb.* extract and standard diclofenac sodium is 61.07% and 70.75%, respectively. Investigation showed that with the increase in concentration, *T. grandiflora* Roxb. has the ability to inhibit protein denaturation significantly and is comparable with the standard. A comparison of the effects of the methanolic extract and standard on the inhibition of egg albumin denaturation is shown in **Fig. 1.**

TABLE 1: *IN-VITRO* ANTI-INFLAMMATORY ACTIVITY OF *T. GRANDIFLORA* ROXB. ON INHIBITION OF EGG ALBUMIN DENATURATION

Concentration (µg/ml)	% Inhibition of standard and mean ±SEM	% Inhibition of sample and mean ±SEM
100	26.88 ± 0.58**	11.32 ± 0.56**
200	39.5 ± 0.32**	22.29 ± 0.76**
300	48.8 ± 0.031**	33.35± 0.48**
400	57.16 ± 0.49**	45.05 ± 0.31**
500	70.75 ± 0.47**	61.07 ± 0.47**

All values represent Mean ± SEM, n=3. The data was analyzed by one-way Analysis of Variance (ANOVA) followed by Dunnett'stest (control v sall), the minimum value of*p<0.05 is considered as significant, **p < 0.01, ***p <0.001.

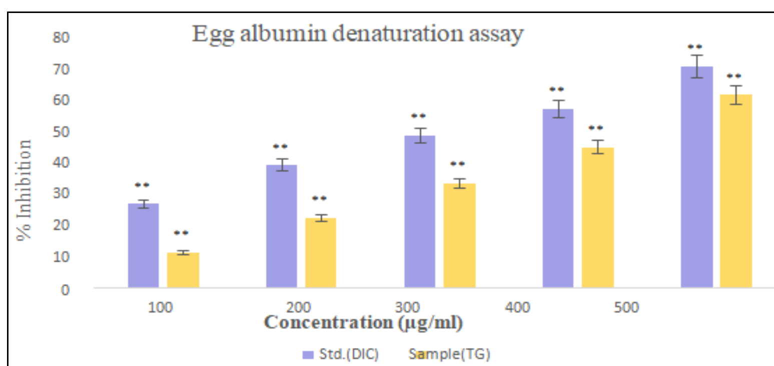

FIG. 1: COMPARISON OF THE EFFECTS OF THE METHANOLIC EXTRACT OF *T. GRANDIFLORA* ROXB. AND STANDARD DICLOFENAC SODIUM ON INHIBITION OF EGG ALBUMIN DENATURATION

Bovine Serum Albumin (BSA) Denaturation Method: The percentage inhibition of BSA albumin denaturation of the extract was compared with the standard Diclofenac sodium.

The study was performed in triplicate; the percentage inhibition and the drug response comparison are given in **Table 2**. The IC_{50} of *T. grandiflora* Roxb. was found to be 400.76 µg/mL as compared to the standard Diclofenac Sodium, which was found to be 267.36 µg/mL. The highest levels of inhibition for standard diclofenac sodium and *T. grandiflora* extract are 75.75% and 57.3%, respectively.

The study revealed that *T. grandiflora* Roxb. is comparable to the standard and significantly inhibits protein denaturation with an increase in concentration, the comparison of which is shown in **Fig. 2.**

TABLE 2: *IN-VITRO* ANTI-INFLAMMATORY ACTIVITY OF *T. GRANDIFLORA*ROXB. ON INHIBITION OF BSA DENATURATION

Concentration (µg/ml)	% Inhibition of standard and mean ±SEM	% Inhibition of sample and mean ±SEM
100	31.4 ± 0.05**	30.43 ± 0.15**
200	41.9 ± 0.22**	36.46 ± 0.05**
300	55.7 ± 0.08**	43.13 ± 0.17**
400	63.4 ± 0.15**	48.79 ± 0.12**
500	75.5 ± 0.20**	57.63 ± 0.05**

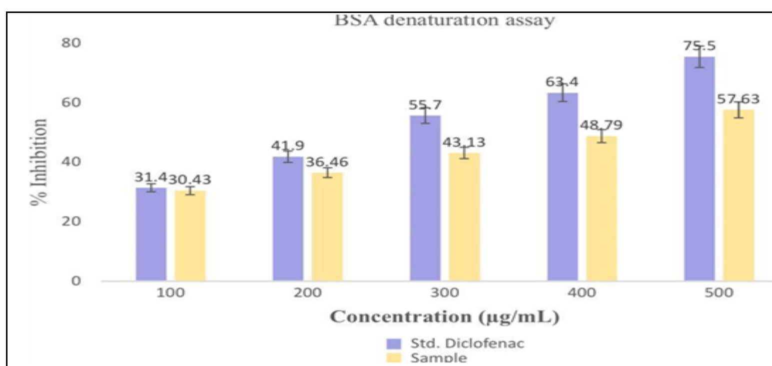

FIG. 2: COMPARISON OF THE EFFECTS OF THE METHANOLIC EXTRACT OF *T. GRANDIFLORA* ROXB. AND STANDARD DICLOFENAC SODIUM ON INHIBITION OF BOVINE SERUM ALBUMIN DENATURATION

***In-vivo* Anti-Inflammatory Activity:**

Carrageenan-induced Pawedema: Acute inflammation caused by carrageenan is one of the best test methods to evaluate anti-inflammatory drugs. Within an hour of the carrageenan injection, the first stage of inflammation sets in and is triggered in part by the stress of the injection and histamine and serotonin [13]. Subcutaneous injection of carrageenan on the rat paws induced edema thus indicating acute inflammation. The paw edema of the different groups of rats following treatment with standard diclofenac sodium and Methanolic extract have been shown in **Table 3.**

The results showed that Methanolic extract 200mg/kg & 400mg/kg b.w. considerably reduced carrageenan-induced paw edema, and their effects were comparable to the standard diclofenac sodium. The anti-inflammatory activity of the extract was found to be dose-dependent. The drug response comparison is shown in **Fig. 3.**

TABLE 3: ANTI-INFLAMMATORY ACTIVITY OF METG ON CARRAGEENAN INDUCED PAW EDEMA *METG- METHANOLIC EXTRACT OF *THUNBERGIA GRANDIFLORA* ROXB

Group	Treatment	Initial thickness	Change in paw thickness in mm (Mean ±SEM) and % inhibition of paw edema				
			1h	2h	3h	4h	5h
I	Control (Dist. H_2O)	3.45±0.15	4.6±0.006	4.75±0.009	4.92±0.1	4.65±0.18	4.27±0.1
II	Standard (DIC)	3.54±0.02	4.34±0.09** 30.43%	4.2±0.009** 53.84%	3.86±0.09** 78.23%	3.86±0.09** 73.33%	3.84±0.007** 63.41%
III	METG 200mg/kg b.w.	3.7 ±0.07	4.58±0.01* 15.65%	4.63±0.03* 29.23%	4.62±0.02* 37.41%	4.54±0.02* 30%	4.29±0.02* 28.04%
IV	METG 400mg/kg b.w.	3.82±0.1	4.75±0.02* 19.13%	4.71±0.01* 31.53%	4.68±0.01* 41.49%	4.58±0.01* 36.66%	4.38±0.01* 31.70%

All values represent Mean ± SEM, n=4. Values in the parenthesis represent % inhibition. The data were analyzed by one-way Analysis of Variance (ANOVA) followed by Dunnett's test (control vs all), the minimum value of *p<0.05 is considered as significant, **p < 0.01,***p< 0.001.

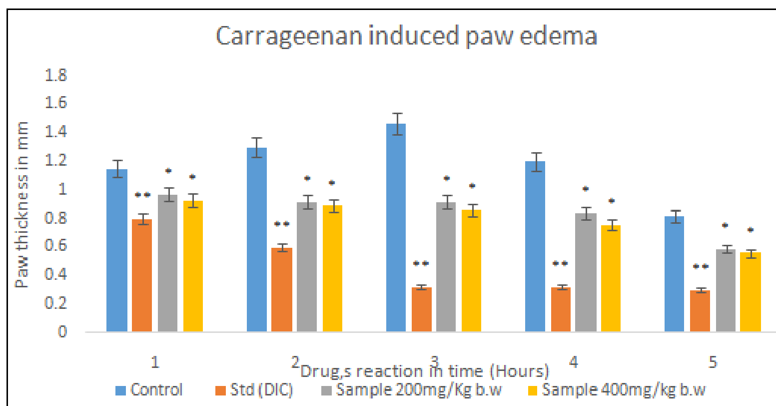

FIG. 3: COMPARISON OF ANTI-INFLAMMATORY EFFECTS OF *THUNBERGIA GRANDIFLORA* ROXB. WITH CONTROL AND STANDARD GROUP

HPTLC Fingerprinting: HPTLC fingerprinting of METG along with standard β-Sitosterol was performed by developing the plate in a Twin trough chamber saturated with the solvent system petroleum ether: ethyl acetate: acetonitrile (8.2: 1.8: 0.1) for 20 minutes. The plates were then scanned at 366 nm and 254 nm. Peaks of sample and standard were observed. The peak table of the chromatogram with different Rf values is shown in **Table 4.** TLC plate under normal light post derivatization and under UV at 366nm post derivatization is shown in **Fig. 4** (**A** & **B**) respectively.

TABLE 4: PEAK TABLE OF THE CHROMATOGRAMS WITH DIFFERENT VALUES *METG-METHANOLIC EXTRACT OF *THUNBERGIA GRANDIFLORA* ROXB.

Track	Assigned substance	Rf
1	β -Sitosterol	0.35
2	β -Sitosterol	0.35
3	METG	0.35
4	METG	0.35
5	METG	0.34
6	METG	0.35
7	METG	0.35
8	METG	0.35

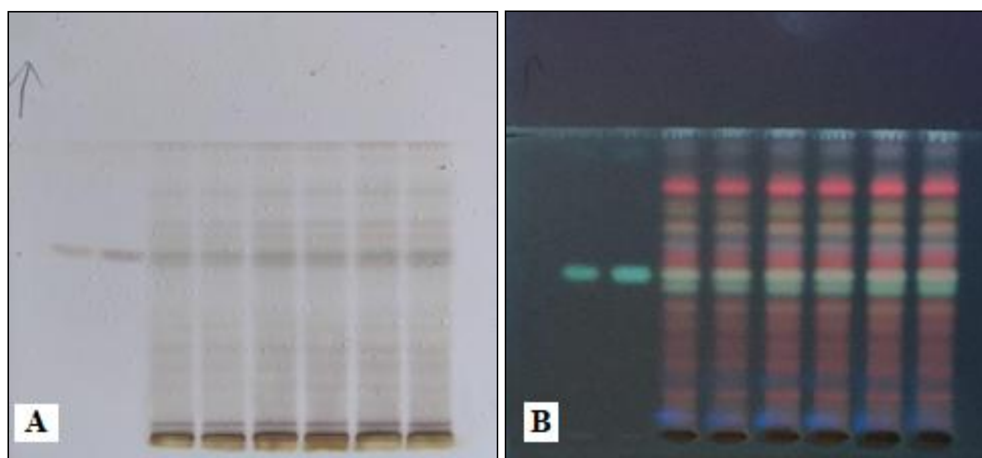

FIG. 4: (A) TLC PLATE UNDER NORMAL LIGHT POST DERIVATIZATION (B) TLC PLATE UNDER UV AT 366nm POST DERIVATIZATION

HPTLC Quantification: HPTLC quantification of METG along with standard β-Sitosterol was performed by developing the plate in a Twin trough chamber saturated with the solvent system petroleum ether: ethyl acetate: acetonitrile (8.2: 1.8: 0.1) for 20 minutes.

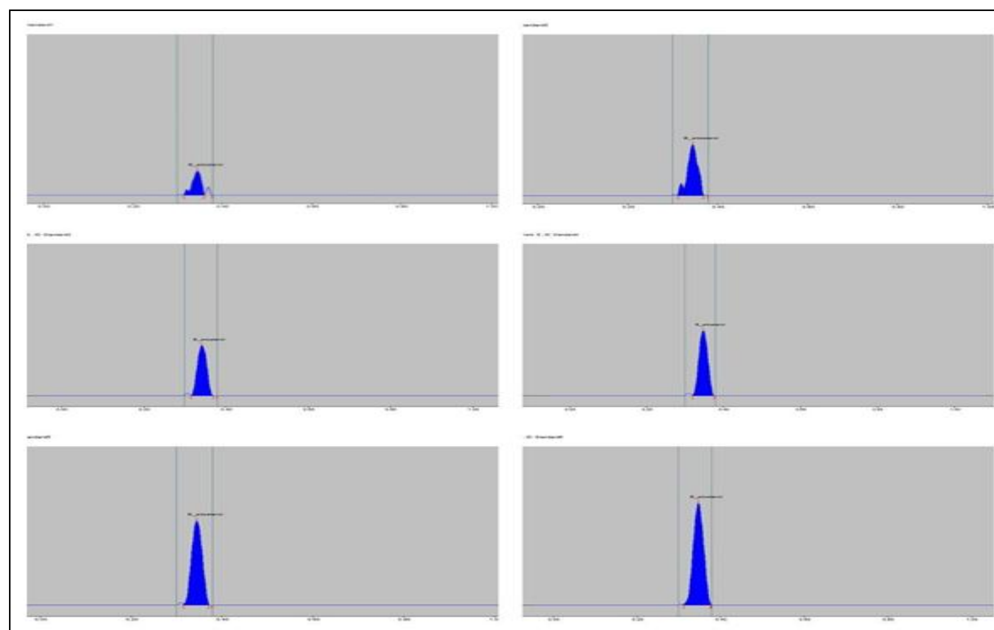

FIG. 5(A); PEAK DIAGRAMS OF STANDARD B-SITOSTEROL AT DIFFERENT STANDARD LEVELS

The plates were then scanned at 366 nm and 254 nm. HPTLC study revealed resolutions of bands of β-Sitosterol at Rf 0.35. The peak diagrams of standard β-Sitosterol at different standard levels & Methanolic extract of the sample at two different concentrations are shown in **Fig. 5** (**A** & **B**) respectively, and the three-dimensional diagram of different peaks **Fig. 6** in the densitogram are shown.

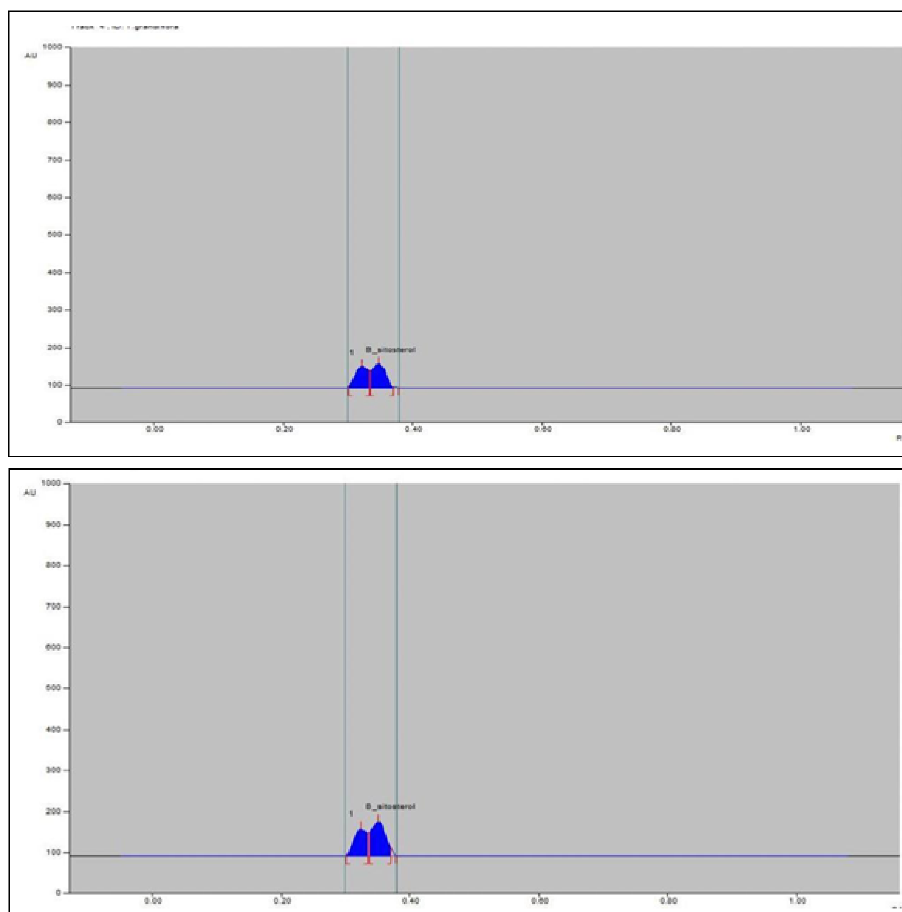

FIG. 5(B): PEAK DIAGRAMS OF METHANOLIC EXTRACT OF SAMPLE AT TWO DIFFERENT CONCENTRATIONS

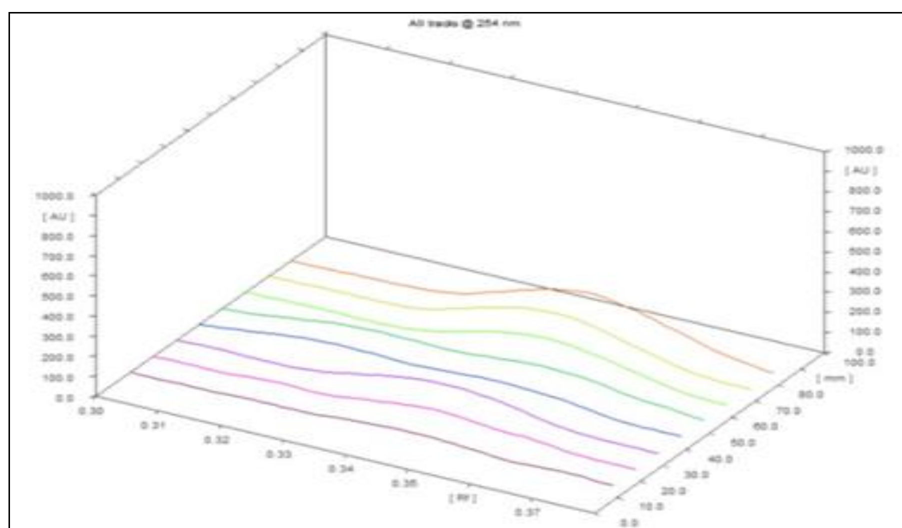

FIG. 6: THREE-DIMENSIONAL DIAGRAM OF DIFFERENT PEAKS IN THE DENSITOGRAM

The identity of the bands of β-Sitosterol in the samples were confirmed by overlapping their UV absorption spectrum with that of the standards. Both the sample and the standard showed similar

λmax as shown in **Table 5**. UV absorption spectrum of standard (β -Sitosterol) and sample is given in **Fig. 7.**

TABLE 5: RF AND MAXIMUM SIGNAL OF Β –SITOSTEROL

Track	Rf	Assigned substance	Maximum signal
1	0.35	β -Sitosterol	399 nm
2	0.35	β -Sitosterol	399 nm
3	0.34	β -Sitosterol	400 nm
4	0.35	β -Sitosterol	406 nm
5	0.35	β -Sitosterol	407 nm
6	0.35	β -Sitosterol	400 nm
7	0.35	β -Sitosterol	400 nm
8	0.35	β -Sitosterol	400 nm

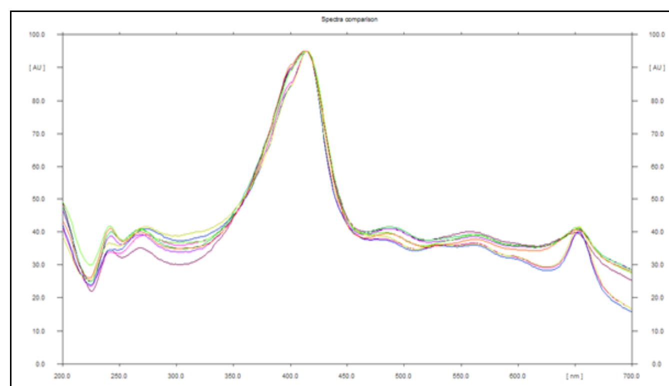

FIG. 7: UV ABSORPTION SPECTRUM OF STANDARD (β -SITOSTEROL) AND SAMPLE METG

Six standard levels (2, 4, 6, 8, 10 and 12µl) β-Sitosterol were spotted along with two samples (8 and 10µl). The calibration curve was plotted using each spot's concentration (µl/spot) and peak height.

Linear calibration curve of methanolic extract is shown in **Fig. 8**. The amount of β-Sitosterol in the sample was calculated using the linear calibration curve, which showed an SD of 1.42%. The calibration result is shown in **Fig. 9.**

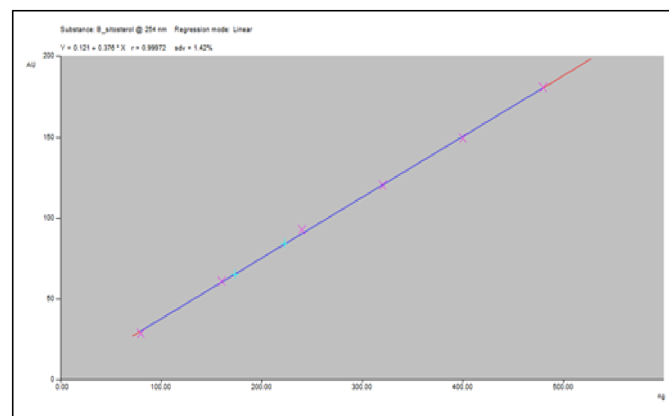

FIG. 8: LINEAR CALIBRATION CURVE OF METHANOLIC EXTRACT

Calibration results per Analysis

Sample from vial 2: T.grandiflora

Result via height

Substance	Rf	X(average)	CV [%]	n	Regression	Remark
B_sitosterol	0.35	198.18 ng	17.686	2	Linear	

FIG. 9: CALIBRATION RESULT

The methanolic leaf extracts of *Thunbergia grandiflora* Roxb. is reported to contain β -Sitosterol of 22 ng/µl.

CONCLUSION: The present study was carried out to study the *in-vitro* and *in-vivo* anti-inflammatory, HPTLC fingerprinting and quantification of the β – Sitosterol from *Thunbergia grandiflora* Roxb.

In this study, three solvents of increasing polarity *i.e.,* petroleum ether, chloroform and methanol were used for successive Soxhlet extraction. The different plant extracts of *Thunbergia grandiflora* Roxb. were successfully extracted using Soxhlet apparatus. Compared to the other extracts, the methanolic extract had the highest yield. This may be due to the solvent's capacity to extract substances with various degrees of polarity. Two *in-vitro* anti-inflammatory studies- Egg albumin and BSA denaturation studies, were performed with the methanolic extract of *Thunbergia grandiflora* Roxb. The results obtained from the anti-inflammatory studies at different concentrations were compared with the standard (diclofenac sodium), which shows marked anti-inflammatory activity. The anti-inflammatory activity was found to be concentration dependent and may be due to the presence of phytoconstituents in the methanolic extracts of *Thunbergia grandiflora* Roxb. The extract may help reduce inflammation. Results from the in-vivo anti-inflammatory study: Carrageenan-induced paw edema showed considerable anti-inflammatory action for both doses (200 and 400mg/kgb.w.) by reducing paw edema. However, further research is required to determine the exact anti- inflammatory mechanism of the plant extract.

The HPTLC Analysis Confirmed the Presence of the Phytosterol: β -Sitosterol. β -Sitosterol is known for curing heart diseases and fighting high cholesterol levels. It is also known to help prevent cancer, allergies, asthma, etc. Further quantification of the methanolic extract of *Thunbergia*

grandiflora Roxb. was done. The amount of β -Sitosterol was 22 ng/μl. We may infer that the presence of β -Sitosterol may play a role in the anti-inflammatory action. According to these findings, the methanolic plant extract has promising anti-inflammatory properties that could, in the future, aid in the formulation of novel anti-inflammatory compounds that are effective, active, and less harmful for a variety of systemic biological processes; further research is necessary.

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CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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